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PRINCIPAL INVESTIGATOR: Arnaud Monvoisin, Ph.D.

CONTRACTING ORGANIZATION: The University of California, Los Angeles
Los Angeles, California 90024-1406

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6. AUTHOR(S) :
Arnaud Monvoisin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, Los Angeles
Los Angeles, California 90024-1406

E-Mail: arnaud@ucla.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Vascular endothelial growth factor(VEGF) is one of the most relevant mediators of capillary recruitment and stimulator of tumor-angiogenesis. Substantial evidence implicates VEGF in the vascularization of tumors, including those related to the prostate gland. Once synthesized and secreted by the normal prostate epithelium, VEGF is frequently bound to extracellular matrix molecules, remaining inaccessible to its receptors present on endothelial cells. Subsequent release of VEGF from extracellular sources is thought to require break-down of matrix proteins by matrix metalloproteinases(MMPs). However, its exact regulation is unknown. We previously found that MMP-3 cleaves $VEGF_{165}$(~22kDa) directly, releasing two major $VEGF_{165}$ cleavage products, ~16kDa and ~6kDa. Here we report that, by western analysis with epitope-specific antibodies, Edman sequencing and MALDI/MS analysis for both fragments to determine the cleavage sites in VEGF, MMP-3 cleaves $VEGF_{165}$, separating the c-terminal heparin binding domain from the rest of $VEGF_{165}$ molecule that contains the receptor binding region. The ~16kDa fragment is functionally active as it phosphorylates VEGFR-2 in porcine aortic endothelial cells at a level comparable by conditioned media from uncleaved $VEGF_{165}$-expressing cells. The data imply that VEGF may be processed extracellulary releasing bioactive fragments and that this proteolysis might offer an important mode for regulation on VEGF bioavailability.

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Introduction

VEGF-A is one of the most relevant mediators of capillary morphogenesis and stimulator of tumor-angiogenesis. VEGF-A exists in five forms, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{205}$, as a result of alternative splicing from a single gene. These various isoforms of VEGF-A differ in their affinity for heparin and extracellular matrix (ECM) proteins. The gene consists of 8 exons and all isoforms share exons 1-5 and exon 8. VEGF dimerization and receptor binding domains are encoded by exons 1-5 and the heparin and ECM binding domains are encoded by exons 6 and 7. Because of the heparin binding domain, all VEGF isoforms except for $VEGF_{121}$ rapidly bind to components of the ECM upon secretion, becoming non-accessible to its receptors located on endothelial cells: VEGFR-1, VEGFR-2 and neuropilin. Release of VEGF from the matrix is essential for stimulation of angiogenesis. It has been thought that VEGF may become available through cleavage of ECM proteins. However, the enzymes that mediate the release of matrix-sequestered VEGF remain to be determined. Recent findings from our laboratory revealed that VEGF might be cleaved directly by extracellular proteases releasing bioactive peptides, a finding that contradicts the dogma that VEGF is released "intact" through cleavage of extracellular matrix proteins. Although both might take place in vivo, our results would argue that VEGF is cleaved directly releasing bioactive fragments and that this proteolysis is an important mechanism involved in its regulation.

Body

Task 1. To determine the MMP3 and MMP9 cleavage sites in VEGF and the sequence of the released VEGF peptides:

Further study on MMP cleavage of VEGF revealed that MMP9 cleavage by VEGF was due to a contamination of MMP3 in MMP9 preparation. Therefore, I only pursued the MMP3 cleavage of VEGF. Previously we found that MMP-3 cleaves $VEGF_{165}$ (~22kDa in SDS-PAGE under reducing condition) directly, releasing two major $VEGF_{165}$ cleavage products, ~16kDa (for glycosylated VEGF, ~13kDa for unglycosylated) and ~6kDa. In an attempt to determine the MMP3 cleavage sites in VEGF and the sequence of the released VEGF peptides, first, the VEGF cleavage products by MMP3 were analyzed by western analysis with epitope specific antibodies. The ~16kDa and the ~6kDa fragments result from the N- and C-termini of $VEGF_{165}$, respectively (Fig. 1).

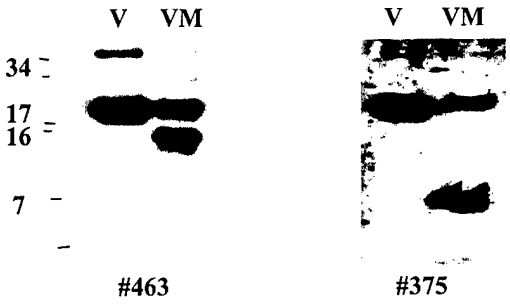
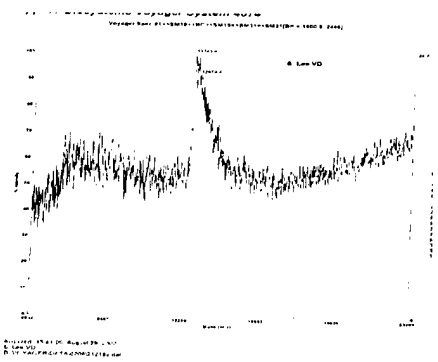


Fig. 1. Detection of the cleaved VEGF fragments by epitope-specific antibodies. VEGF and MMP-3 were incubated at 37°C for 10h with 1:1 molar ratio. VEGF Cleavage was examined by SDS-PAGE followed by immunoblotting with epitope-specific antibodies (#463, specific to N-terminus of VEGF, #375, to C-terminus).

Then, to locate the cleavage sites in VEGF, 1) the ~16 and ~6kDa fragments were separated by SDS-PAGE and both were subject to N-terminal Edman sequencing, 2) the ~13kDa (unglycosylated VEGF was used to avoid heterogeneity due to glycosylation) fragment was separated by SDS-PAGE and purified from the gel for MALDI/MS (Fig. 2), LC/MS analysis, and 3) the ~13kDa fragment was separated by SDS-PAGE and subject to in gel digestion followed by LC/MS/MS (data not shown). These results indicated that the cleavage occurs proximal to the end of exon 5. These were further

confirmed by using fluorescence-labeled peptide composed of amino acids 103-119 which contain the candidate cleavage sites (Fig. 3A, B). We made peptide where coumarin fluorescence is linked to its N-terminus and DNP quencher to its C-terminus. When this peptide was incubated with different molar ratio of MMP-3, dose response increase of fluorescence signal was detected (Fig. 3C). Since VEGF dimerization and receptor binding domains are encoded by exons 1-5 and the heparin and ECM binding domains are encoded by exons 6 and 7, these results demonstrate that MMP-3 cleaves $VEGF_{165}$, separating the c-terminal heparin binding domain from the rest of $VEGF_{165}$ molecule that contains the VEGFR-1 and VEGFR-2 binding region. Currently, we are generating mutant VEGF165 and fluorescence-labeled mutant peptide that are labile to MMP3 proteolysis.



Fig,2 MALDI/MS analysis of the ~13kDa fragment.

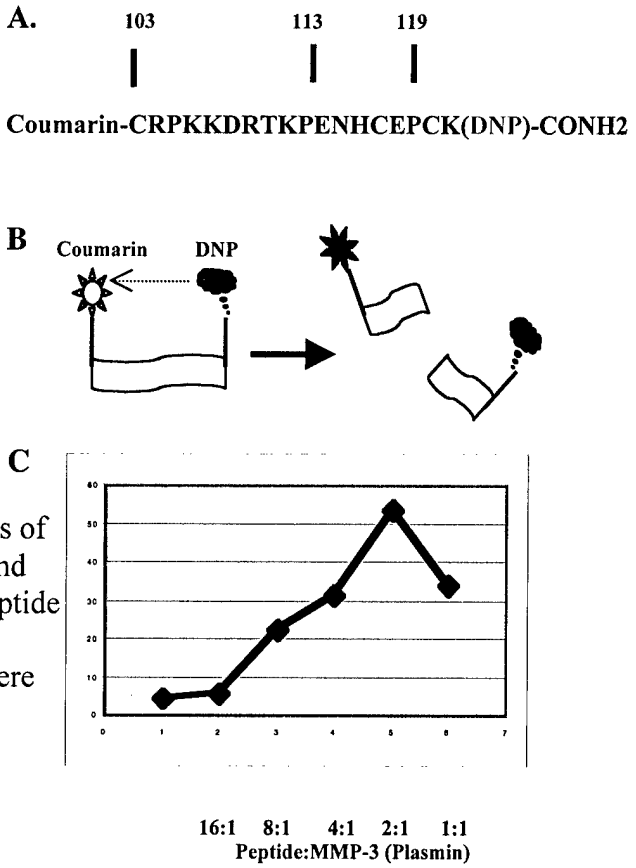


Fig. 3 Fluorescence labeled peptide. A. N-terminus of peptide is conjugated to Coumarin (fluorescence) and C-terminus to DNP (quencher). B. Cleavage of peptide will lead to separation of coumarin and DNP and to subsequent fluorescing by coumarin. C. Peptides were incubated at 37°C for 2h with different molar ratio. Fluorescence signal was detected by fluorometer.

Task. 2 To determine the relevance of released peptides to VEGF receptor signal transduction:

Since the amino acid 113 in VEGF165 is the most proximal end of exon 5, expression vectors for VEGF113 was generated and stably transfected into 293T cells to make the ~16kDa VEGF fragment. To test the ability of VEGF peptides to result in receptor phosphorylation, conditioned media of VEGF113 expressing cells were incubated with porcine aortic endothelial cells overexpressing VEGFR2 (PAE-KDR). We found that VEGF113 (the ~16kDa VEGF fragment) is functionally active as it phosphorylates VEGFR-2 in porcine aortic endothelial cells at a level comparable to VEGFR-2 phosphorylation by conditioned media from cells expressing full length uncleaved $VEGF_{165}$ (Fig. 4). The ability of VEGF113 on endothelial cell proliferation, survival, angiogenesis and its binding to

receptor will be tested by using purified VEGF113. Maxi-preparation of VEGF113 and subsequent purification is underway.

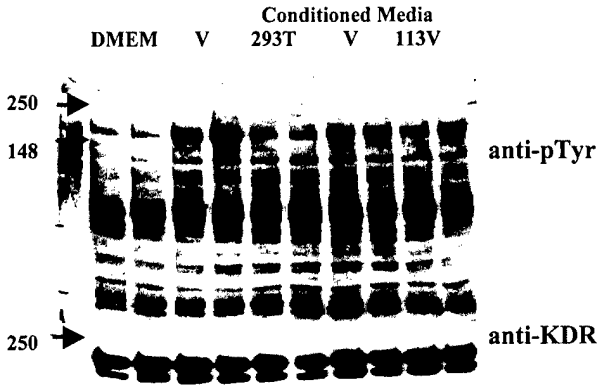


Fig. 4. Phosphorylation of KDR (VEGFR-2) by VEGF fragment. PAE- KDR cells were incubated with purified VEGF, conditioned media from wild type VEGF and cleaved VEGF expressing cells. Phosphorylation of VEGFR-2 was examined by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine and anti-KDR antibodies, respectively.

As a pilot study to identify MMP3-cleaved VEGF peptides in vivo, we first tested the battery of VEGF antibodies (1 polyclonal, 3 epitope specific) to immunoprecipitate the ~16kDa VEGF fragment. The polyclonal antibody (provided by Dr. Don Senger, Harvard University) was able to detect ~16kDa fragment from tumor lysates of T47D cells expressing full length mVEGF xenograph. We will use this specific antibody to identify MMP3-cleaved VEGF peptides from prostate cancer cell lines.

Key Research Accomplishments

Task 1.

Characterization of VEGF peptides by western analysis
Obtaining mass spectrometry information of VEGF peptides

Task 2.

Generation of expression vectors for VEGF113 (~16kDa fragment)
Generation of stable cells overexpressing VEGF113
Determination of the ability of VEGF113 to lead to receptor phosphorylation
Optimization of condition to identify ~16kDa VEGF peptides in vivo

Reportable Outcomes

Development of cell lines

Conclusions

These results demonstrate that MMP-3 cleaves $VEGF_{165}$, separating the c-terminal heparin binding domain from the rest of $VEGF_{165}$ molecule that contains the VEGFR-1 and VEGFR-2 binding region. The ~16kDa VEGF fragment is functionally active as it phosphorylates VEGFR-2 in porcine aortic endothelial cells at a level comparable to VEGFR-2 phosphorylation by conditioned media from cells expressing full length uncleaved $VEGF_{165}$. Overall the data imply that VEGF may be processed extracellulary releasing bioactive fragments and that this proteolysis might offer an important mode for regulation on VEGF bioavailability.

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